Tampere University

Sonja Nousiainen

DEVELOPING AN IN VITRO STROKE MODEL FOR STUDYING ADIPOSE STEM CELLS' PARACRINE EFFECT ON NEURONAL RECOVERY AFTER ISCHEMIA

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Abstract

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Author: NOUSIAINEN, SONJA KATARIINA
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Supervisors: Associate professor Susanna Miettinen & MSc Miia Juntunen
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Stroke is one of the leading causes of mortality worldwide. Studies aiming to find new therapeutic methods improving patient's outcome afterwards are conducted in the field of stem cell research. Adipose stem cells (ASC) may possess neuroprotective ability and they have shown a potential for helping neuronal cells suffered from ischemic injury to regenerate. The aim of the present research is to develop an in vitro ischemia model for studying ASC possible paracrine effect on damaged neuronal cells. Neuronal cells used in this study were differentiated from human neuroblastoma cell line, SH-SY5Y cells. In vitro ischemia was caused by oxygen-glucose deprivation (OGD) treatment.

Neuronal cell differentiation protocol for SH-SY5Y cells was tested, and results verified with immunocytochemistry using antibodies targeted to mature neuronal cell markers. Differentiated SH-SY5Y cells were predisposed to ASC environment with different culture media and in co-cultural system. Neuronal cell proliferation in co-cultural system was measured using CyQUANT-analysis and neuronal cells were also examined with immunocytochemical assay. Four-hour OGD treatment was conducted to neuronal cell culture and effect was examined using immunocytochemistry with apoptosis marker.

Protocol used for SH-SY5Y neuronal differentiation was successful and cells expressed mature neuronal markers based on immunocytochemistry and fluorescence microscoping. CyQuant analysis showed that SH-SY5Y cells survived well in co-cultural system. OGD treatment time was too short and SH-SY5Y derived neuronal cells remained viable. Also, apoptosis marker was not visible with immunocytochemistry after the OGD treatment.

ASCs´ paracrine effect on neuronal cells after ischemic injury can be studied with human origin SH-SY5Y derived neuronal cell culture as they survived in the co-cultural system. In the present study, too short OGD treatment time prevented examination of the regenerative paracrine effect of ASCs on injured neuronal cells as cells remained intact. Prospective studies could examine different OGD-treatment times to cause ischemic injuries on neuronal cells in order to study if neuroprotectivity is caused by the presence of ASC or the secrete products.

Keywords: adipose stem cell, in vitro ischemia, neuronal differentiation, SH-SY5Y, stroke

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Iskeemisen aivoinfarktin hoitovaihtoehdot ovat rajalliset ja uusia hoitomuotoja tutkitaan jatkuvasti. Kantasoluhoidoista on toivottu uuttaa menetelmää aivoinfarktin jälkeisten halvausoireiden hoitoon. Aikuisen rasvan kantasoluilla on havaittu olevan iskeemisen hermosoluvaurion korjautumista ja solujen uusiutumista vaurion jälkeen tukevia parakriinisia ominaisuuksia. Tämän tutkimuksen tavoitteena on kehittää in vitro iskemia -malli SH-SY5Y peräisistä hermosoluista rasvan kantasolujen parakriinisten vaikutusten tutkimiseksi.

Hermosolukko erilaistettiin ihmisperäisistä SH-SY5Y soluista. Erilaistuminen varmistettiin immunosytokemiallisilla menetelmillä käyttämällä kypsissä hermosoluissa esiintyvien proteiinien vasta-aineita. Hermosolukko altistettiin erilaisille kasvatusolosuhteille rasvakudoksen kantasolujen sekä niiden viljelyliuosten kanssa, jotta rasvan kantasolujen parakriinisia vaikutuksia hermosoluihin voitaisiin tutkia yhteisissä kasvatusolosuhteissa. Hermosolujen selviytymistä erilaisissa kasvatusolosuhteissa tutkittiin solujen määrään korreloivalla, fluoresenssia mittaavalla CyQUANT-analyysillä. Osalle yhteisviljelmän hermosoluja aiheutettiin iskeeminen vaurio happi-glukoosideprivaatiolla. Vaurioitumista tutkittiin immunosytokemiallisella menetelmällä käyttämällä apoptoositekijään sitoutuvaa vasta-ainetta.

SH-SY5Y solut saatiin onnistuneesti erilaistumaan hermosolujen suuntaan. Immunosytokemiallisessa tutkimuksessa tulivat esiin kypsien hermosolujen proteiinit. CyQuant-analyysin perusteella hermosolukko selvisi hyvin yhteisviljelyolosuhteista rasvan kantasolujen sekä niiden viljelyliuosten kanssa. Iskeemisen vaurion aiheuttaminen ei onnistunut, koska hypoksiaan käytetty aika saattoi olla liian lyhyt. Apoptoositekijä ei tullut näkyviin immunosytokemiallisessa tutkimuksessa.

Onnistunutta protokollaa hermosolukon erilaistamisesta ja sen todennettua selviämistä rasvan kantasolujen kanssa yhteisviljelmässä voidaan hyödyntää jatkotutkimuksissa. Hypoksia-aika tulisi kuitenkin optimoida vaurion esiin saamiseksi, jotta rasvakudoksen kantasolujen parakriinisia vaikutuksia hermosolukon iskeemiseen vaurioon voitaisiin tutkia.

Avainsanat: aivoinfarkti, hermosolukon erilaistaminen, in vitro iskemia, kantasolu, SH-SY5Y

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Table of Content

Abbrevations

ASC	adipose stem cell
ASC-CM	adipose stem cell conditioned medium
BDNF	brain-derived neurotrophic factor
DAPI	4′,6-diamidino-2-phenylindole
db-cAMP	dibutryl cyclic adenosine mono-phosphate
DM#1	differentiation media 1
DM#2	differentiation media 2
DM#3	differentiation media 3
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EMEM	Eagle's minimum essential medium
FBS	fetal bovine serum
HPL	human platelet lysate
KCl	potassium chloride
MAP2	microtubule-associated protein 2
MSC	mesenchymal stem cell
OGD	oxygen-glucose deprivation
PBS	phosphate buffered saline
P/S	penicillin/streptomycin
RA	retinoic acid

1 REVIEW OF LITERATURE

1.1 Stroke

Stroke as one of the noncommunicable disease is among the leading causes of mortality worldwide. Stroke is in the second place of top 10 causes of death in 2016 around the world with all ages and both sexes with 5,781 million deaths. (1)

In stroke, progressive or sudden obstruction of a large cerebral artery results to reduced cerebral blood flow. Collateral blood supply in brain can maintain a normal perfusion within the area of obstructed artery for some time. But by the time, resistance vessels reach their capacity to regulate to changed perfusion, metabolism in the area starts to change and hypoxic conditions may develop. The result can be ischemic damage of neuronal cells in the brain area obstructed artery serves. These events can lead to stroke. (2)

Several causal risk factors can result to event of ischemic stroke in individual. Hypertension is one significant risk factor for all regions despite that risk factors vary regionally (countries, sexes, age, ethnic groups). Other important risk factors are smoking, alcohol consumption, diabetes mellitus (3) and obesity and overweight (4). Risk for ischemic stroke grows in both sexes within the age (5) .

Thrombolytic therapy with tissue plasminogen activator (t-PA) is a current treatment option for stroke patients but t-PA has its limitations due to narrow time window for treatment and complications such as intracerebral hemorrhage (6-8). Regenerative, stem-cell based therapies for ischemic stroke has been studied in recent years with both animal and cell culture models with promising results. There have also been few clinical trials. Although stem-cell based therapies show potential for treating stroke, more evidence is needed in order to develop more reliable therapeutic approaches. (8-11)

1.2 Adipose Stem Cells

Stem cells are undifferentiated cells that are capable to specialize to multiple cell lineages in response to cytokines, hormones and other chemical agents. Adult stem cells are multipotent or unipotent stem cells found from adult tissues, such as bone marrow, skin or adipose tissue. (13) Adult multipotent

mesenchymal stem cells (MSC) can be used in stem cell studies in the field of regenerative medicine research. MSCs can be harvested from different sources, and in studies commonly used sources are bone marrow or adipose tissue. Adipose stem cells (ASC), harvested from adipose tissue, might possess a preferable source for ischemic cell therapy studies. ASCs are easier to harvest comparing to MSCs harvested from bone marrow. ASCs are also more proliferative and for example secrete more growth factors and have superior paracrine function comparing to bone marrow-derived MSCs. (11,14,15)

ASCs´ paracrine effect have been examined in animal models. Studies suggests that ACS administration after ischemic injury can improve the outcome after the treatment and on cellular level reduce the infarct site (16). Also, the neuroprotective effect of ASCs is maintained at least three weeks. Thus, ACSs have a potential for regeneration and repair of damaged tissues, and they may possess an ability to improve neuronal injury regeneration. (17,18)

ASCs may have an impact on neuronal recovery and neuroprotective effects through trophic factors released from ASCs in the damaged tissue (10). Few studies suggest that ASCs´ effect for neuronal regeneration after neuronal tissue damage is mediated through brain-derived neurotrophic factor (BDNF) secreted by ASCs (18-20). Other studies show that several agents might affect the recovery and the treatment could be done with cell-free secrete of ASCs, ASC-conditioned medium (ASC-CM), rather than with whole cell treatment. (21,22)

1.3 SH-SY5Y neuroblastoma cell line and neuronal differentiation

SH-SY5Y (ATCC® CRL-2266™) is a cell line from 1970 and it is subline for parental line SK-N-SH (ATCC® HTB-11™) which has been subcloned for three times. SK-N-SH was established from a metastatic bone tumor in 1970. (23) SH-SY5Y cells can be differentiated into mature neurons and after differentiation cells are post-mitotic and they present mature neuronal cell factors, such as microtubule-associated protein 2 (MAP2). SH-SY5Y cell line is human origin and it has a stable karyotype. Also, karyotype is close to normal human cell, modal number being 47. (24-26)

Original SK-N-SH cell line includes two different phenotypes; substrate-adherent, epithelial S-type and neuroblastic N-type. SH-SY5Y cell line includes small amounts of S-type with majority being N-type. (27) N-type can be differentiated into neuronal cells whereas S-type is considered being

epithelial like and it lacks expression of markers characterized for catecholaminergic neuronal like cells. (26,27)

In undifferentiated form, SH-SY5Y cells show non-polarized look with few blunt projects. Proliferating cells form clumps and cell culture includes both floating and adherent cells. When differentiated, projects are elongated, and cells form network of contacts with neighboring cell clusters. (26)

Differentiation of SH-SY5Y cells to mature neuronal cell population includes serum deprivation with use of retinoic acid (RA), BDNF, dibutryl cyclic adenosine mono-phosphate (db-cAMP), B-27 and potassium chloride (KCl). During differentiation, cells are predisposed to changing content of culture medium. (25)

Common substance used for neuronal differentiation of SH-SY5Y cells is RA. (25) RA promotes neurite outgrowth and neuronal cell survival. For SH-SY5Y neuroblastoma cells RA increases the number of processes as well as elongates them but decreases cell proliferation. (25-30)

Neurotrophins such as BDNF are substances that promote neuronal cell survival and can be used with RA on cell cultures. BDNF is used for supporting mature neuronal cell population. Treatment of SH-SY5Y cell culture with RA and BDNF potentially leads to expression of mature neuronal markers such as MAP2. (28) If neuronal cell culture lacks BDNF, cells may enter the cell cycle and can proceed to apoptotic cell death. (27)

Db-cAMP promotes SH-SY5Y cells morphological differentiation to noradrenergic neuronal phenotype. SH-SY5Y response to db-cAMP by increased intracellular cAMP level and cAMP-depended protein kinase (PKA) pathway leads to neuronal differentiation, growth of neurites and connections between neighboring cells. (31,32)

B-27 is a serum-free supplement used in neuronal cell cultures to improve long-term viability of cells. (33,34) KCl causes cell membrane depolarization and influx of calcium. Increased levels of intracellular calcium trigger a signaling pathway leading to activation in certain gene expression promoting neuronal cell maturation and growth, such as synapse development and dendritic elongation. (35,36)

1.4 In vitro ischemia modelling

In vitro ischemia modelling is done by exposing cultured cells to ischemic condition by removing oxygen and glucose, a method called oxygen-glucose deprivation (OGD). This removal can be performed with enzymes and chemicals causing chemical hypoxia and transforming glucose into unusable structure. Other method is to replace normal cell culture medium with N_2/CO_2 equilibrated medium and incubate cell culture in hypoxic chamber in the absence of glucose. After OGD cells are maintained under normoxid conditions for reoxygenation to observe injuries. (37-39) In vitro studies examine neuronal ischemia primary on neuronal cell cultures but also on brain slices or organotypic cultures are used (40).

1.5 Cell therapies for stroke

Regenerative, stem-cell based therapies for ischemic stroke has been studied in recent years with both animal and cell culture models and also in few clinical trials. Though animal models have shown encouraging results, clinical trials have not been as successful. (8) Clinical trials have shown that administration of stem cells can be safe but the level of efficacy of the stem cell treatment or the improving effect are not yet achieved. (11,41)

Comparing stem cells from different sources, MSCs have shown promising results in studies examining effects in vitro. Though MSCs can be harvested from several sources from adult tissues, especially bone marrow-derived MSCs are widely examined, and results indicate that cells possess neuroprotectivity and neurogenic effect on neuronal cells suffering from ischemic injury. (42) Also, ASCs possess mainly the same abilities as MSCs. What is more, ASCs have higher proliferation capacity and they also produce greater amounts of growth factors. Both in vivo and in vitro exams supported this conclusion. (14)

Clinical trials have shown that stem cell -based therapies are still experimental though laboratory findings support especially MSCs properties supporting neuronal cell recovery. Stem cell-based methods treating stroke are considered as potential therapeutic method for patients with neurologic deficits. Cell therapy outcome in stroke needs to be studied further before assessing new experimental method as a treatment option. (9,11) Biggest concerns using stem cells as a therapeutic method include finding a proper transplantation route, timing and dose of stem cell transplant and to sort out the best source for stem cells used for treatment. (10) Monitoring the response of the brain for the

treatment in vivo is crucial in clinical trials in order to examine the effect and to develop the treatment. (43)

Developing cellular therapy, and developing the products used for cellular therapies requires potency assays especially from early-stage development. Evaluating the potency of cellular treatment product is crucial in order to define the parameters important to the effectiveness of the cellular treatment product and to control the quality of the product. Potency assays gather information mostly from in vitro studies and potency assays should offer relevant data of biologic effects of the processed product. (44)

Cellular product containing ASC might be a treatment option for stroke in the future but more studies, both in vitro and in vivo, need to be performed. Studies examining stem cell treatment in vivo on animal models have met limitations such as tumor formation, cell migration and problems with cell engrafting despite the promising results from in vitro studies. Future studies could aim to better understanding of the mechanism of action in stroke treatment with stem cells. (10) The mechanism of action can be studied with cell cultures in vitro.

2 OBJECTIVES

Hypothesis is that differentiated SH-SY5Y cells can offer a model for studying ischemic stroke effect on neuronal cells in vitro when exposed to OGD. The aim is to differentiate mature neurons from SH-SY5Y cells and to develop an in vitro stroke model with OGD treated differentiated SH-SY5Y cells. This study also investigates if differentiated SH-SY5Y cells survive in co-cultural system with ASCs and ASC-CM in order to study ASC possible paracrine effect on damaged neuronal cells after ischemic event in vitro. To achieve these objectives, following steps were set:

1. Functional differentiation protocol for SH-SY5Y cells to produce mature neuronal cells.
2. Observing if differentiated SH-SY5Y cells survive in co-cultural system with ACS and ACS-CM in order to exclude ASCs` possible impairing effect on neuronal cells growth and proliferation.
3. Causing in vitro ischemia to neuronal cells with 4-hour OGD treatment and observing the level of damage.

ASCs paracrine effect on neuronal cell recovery after OGD can be studied if OGD treatment, differentiation and co-culture system are all successfully conducted. In vitro stroke model with human cells can offer tools for studies developing new therapeutic methods for neurodegenerative disorders, such as cell-based restorative therapies for stroke.

3 MATERIALS AND METHODS

Used materials were provided by Adult Stem cell group and some specific reagents for neuronal differentiation by Neuro Group, both from Faculty of Medicine and Health Technology, Tampere University.

3.2 SH-SY5Y culture and differentiation

SH-SY5Y cells were obtained from ATCC®. At the beginning, the passage of the cells was 27. The vial was thawed and handled according to the manufacturer's instruction (23). Thawed, undifferentiated cells were cultured in T-75 flasks in Eagle's Minimum Essential Media (EMEM) supplemented with 1% penicillin/streptomycin (P/S) and 15% fetal bovine serum (FBS) supplements. Cell culture was maintained in CO_2-incubator at atmosphere of 5% CO_2 and 95% air. Prior differentiation of SH-SY5Y, cells were cultured and passaged for 27 days and during culturing time passage number increased from 27 to 31. Part of cultured cells were storage in the freezer for later use. Reagents used for culture, freezing and differentation are presented in Table 1.

Table 1. Used reagents and differentiation media components for SH-SY5Y cells.

Reagent	**Details**		**Manufacturer**
EMEM	Eagles Minimum Essential Media		ATCC, USA
FBS	Fetal Bovine Serum		Life Technologies (Gibco) Europe BV, Netherlands
P/S	Penicillin/Streptomycin		Lonza Walkersville, USA
Tryple	Includes trypsin, for re-attachment		Life Technologies (Gibco) Europe BV, Netherlands
DMSO	Dimethyl sulfoxide, for freezing cells		Merck KGaA, Germany (Sigma-Aldrich)
Neurobasal	Growth medium for neuronal cells		Life Technologies (Gibco) Europe BV, Netherlands
GlutaMAX	Includes L-glutamin		Life Technologies (Gibco) Europe BV, Netherlands
DPBS	Dulbecco's phosphate buffered saline		Lonza Walkersville, USA
Component		**Stock**	
10 μM RA	All-trans retinoic acid (300,44 g/mol), suspension of 50 mg RA in 33,3 ml 95% EtOH	5 mM	Merck KGaA, Germany (Sigma-Aldrich)
20 mM KCl*	Potassium Chloride	2,5 M	Merck KGaA, Germany (Sigma-Aldrich)
2 mM db-cAMP*	Dibutyryl cyclic adenosine-mono-phosphate	0,1 M	Merck KGaA, Germany (Sigma-Aldrich)
50 ng/ml BDNF*	Brain-derived neurotrophic factor	25 μg/ml	R&D Systems Inc. a Bio-Techne Brand, USA
1x B-27	Neuronal cell culture supplement		Life Technologies Europe BV, Netherlands
Laminin	Laminin coat for wells	100 μg/ml	BioLamina AB, Sweden

*component provided by Neuro Group and/or stock concentration may vary from Shipley & al. (2017) protocol.

3.2.1 Culturing SH-SY5Y cells

Undifferentiated, freezed SH-SY5Y cells were thawed in 37° C degree waterbath for two minutes. After thawing, cells were removed to colonial tube that included 9 ml of basic growth medium (15% FBS). Suspension was centrifuged in 130 g for 6 minutes. Supernatant was aspirated off and cell pellet was re-suspended with 3 ml of basic growth media. Cell-suspension was then pipetted into three 1 ml aliquots and each aliquot transferred into T-75 flasks, each flask containing 9 ml of basic growth medium. Total culture volume in each T-75 flask was 10 ml.

Cell cultures were monitored every day from Monday to Friday to follow-up attachment, viability and confluence rate. Medium was changed when necessary. Medium change was proceeded aspirating off old medium and added fresh basic growth medium which was first warmed into 37° C. When confluence reached 60% to 70%, cells were passaged.

3.2.2 Passage

First, basic growth medium was warmed in 37° C waterbath. Cell cultures in T-75 flasks were monitored and moved into laminar hood. Old medium was aspirated off carefully and 10 ml of Dulbecco's phosphate buffered saline (DPBS) was added to rinse cells. DPBS was aspirated off and 3 ml of Tryple was added into each flask for reattachment. Cells were incubated with Tryple 3 minutes in 37° C, 5% CO_2 incubator. Re-attachment was confirmed under the microscope.

After successful re-attachment 10 ml of warmed basic growth media was added into each flask. Cell-suspensions were collected from the flasks to 50 ml colonial tube and centrifuged in 1000 g for 2 minutes. After centrifuging, supernatant was aspirated off and the pellet was resuspended in 5 ml of basic growth medium.

Cells were count in Bürker-chamber. New T-75 flasks were obtained, and cells plated in density of 1,5-2 million cells in total volume of 10 ml per each T-75 flask.

3.2.3 Freezing aliquots

Before freezing aliquots, cells were cultured and handled according to protocols described above. For freezing, cells were first monitored, re-attached with Tryple, collected into colonial tube, centrifuged and resuspended into 5 ml of warmed basic growth media for counting. Each aliquot for freezing contained 2-5 million cells in volume of 1 ml of basic growth medium supplemented with 5% dimethyl sulfoxide (DMSO).

After counting cells, the needed volume of cell-suspension was pipetted into colonial tube and centrifuged. Supernatant was aspirated off and the pellet resuspended in prepared basic growth medium supplemented with 5% DMSO.

Cell-suspension was distributed into cryo-vials, total concentration in each vial being 2-5 million cells/1 ml. Cryo-vials were placed into -80° C freezer for 24 hours and after that transferred to gas phase nitrogen to storage.

3.2.4 Differentation protocol

First differentiation test was done with SH-SY5Y cells at passage 31 with intent to test the neuronal differentiation protocol. SH-SY5Y cells were plated on day 0 in density of 11 000 c/cm^2 into 6 well plates.

The second differentiation was done with SH-SY5Y at passage 29. On day 10 at the second differentiation test, SH-SY5Y cells were transferred into 24-well plates to carry through differentiation on plates, which were later used to perform co-culture with ASCs and OGD treatment.

Differentiation protocol lasted totally 18 days. Days of action during differentiation protocol are described in figure 1. On intervening days cells´ viability was examined under microscope.

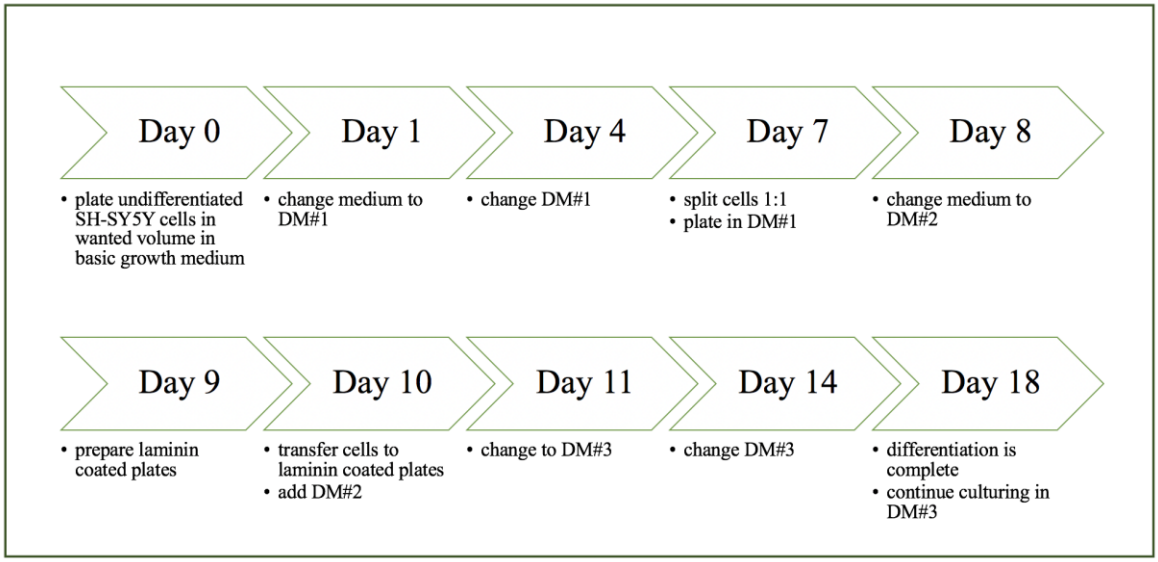

Figure 1. Days of action during differentiation protocol (modified from Shipley et al., 2017 by Juntunen).

During culture and differentiation, medium used for SH-SY5Y cells was changed to fresh medium approximately every third day. Differentiation media were prepared mostly based on instructions in Shipley et al. protocol. Media were prepared beforehand but DM#3 was prepared right before use. Also, RA was added to media right before use.

Basic cell culture for SH-SY5Y cells was performed in EMEM supplemented with 15% FBS and 1% P/S. First differentiation medium DM#1 was EMEM supplemented with 2,5% FBS and 0,2% RA and DM#2 was EMEM supplemented with 1% FBS, 1% P/S and 0,2% RA. DM#3 was serum free in Neurobasal supplemented with 0,2% RA, 0,5% BDNF, 0,2% db-cAMP, 2% B-27, 1% P/S, 1% glutamine and 2% KCl. (Table 2)

Table 2. Media contents used for SH-SY5Y culture and differentiation.

Basic growth media	15% FBS, 1% P/S in EMEM
DM#1	2,5% FBS, 1% P/S, 0,2% RA in EMEM
DM#2	1% FBS, 1% P/S, 0,2% RA in EMEM
DM#3	2% B-27, 2% KCl, 1% P/S, 1% GlutaMAX, 0,5% BDNF, 0,2% db-cAMP, 0,2% RA in Neurobasal

DM=differentation medium

Differentation started on day 0, when cells were plated into 6-well plates. Cultured and maintained SH-SY5Y cells from T-75 flasks were rinsed with DPBS, re-attached with Tryple, centrifuged and counted following the same protocol as described in chapter 3.2.2.

After counting the cells, plating suspension was made. Density for 6 well plate per one well was 100 000 cells in 2 ml total volume of basic growth media. Totally, 600 000 cells per each 6 well plate were plated, total volume being 12 ml per whole plate. Cultured plates were put into incubator (37°C, 5% CO_2).

On day 1 in differentation protocol, basic growth medium was aspirated off and changed to DM#1 (2,5% FBS). DM#1 was prepared beforehand, but RA was added right before using the medium. Days 2 and 3 were intervening days and cells were monitored during these days.

On day 7, cells were divided in 1:1. Cells were re-attached from 6-well plates with Tryple. Old medium was aspirated off and 500 µl of Tryple was added into each well. Plates were then positioned into incubator (37°C, 5% CO2) for 3 minutes. However, cells were not properly detached, so another 200 µl of Tryple were needed and plates were incubated for extra 2 minutes in incubator for proper detachment.

After cells were detached, 2 ml of DM#1 was added into each well and cells were collected into one colonial tube. Cell suspension was then distributed to new 6-well plate, 2 ml of cell suspension into each well.

On day 8, new DM#2 (1% FBS) was prepared but again, without RA, which was added right before use. DM#1 was replaced with fresh DM#2, 2 ml per each well.

Laminin coated plates were prepared on day 9. Used plate was 24 well plate where one well area is 1,9 cm^2. Laminin concentration for 24-well was 15 μg/ml. The need of laminin solution was counted. Stock concentration of 100 μg/ml and totally 256 μl volume of PBS diluted laminin solution was added to each well. Plate was left overnight to 4°C.

On day 10, cells were transferred into 24 well laminin coated plate in DM#2. Cells were re-attached from 6 well plate with Tryple and cell-suspension was collected into colonial tube adding 2 ml of DM#2 into each well for collection. After centrifuging (1000 g, 2 min) supernatant was aspirated off and cell pellet re-suspended into 2 ml of DM#2. Cells were counted and plated into density of 47500 cells in 1 ml volume per one well on 24-well plate.

On day 11, culture medium was changed to DM#3 (serum free), which was prepared right before use and not in advance. On day 14, new fresh DM#3 was changed for cells. On intervening days 12, 13, 15, 16 and 17 cells were monitored under microscope. Cells were ready for further use in studies on day 18, when differentation of SH-SY5Y cells to mature neuronal cells was completed.

3.3 SH-SY5Y and ASC co-culture system

Successful differentiation was verified with immunocytochemistry described in chapter 3.5. Differentiated SH-SY5Y cells were cultured one week in three different set-ups to observe if neuronal cells survive under condition where they are exposed to ASCs and their culture media.
First group was cultured in basic ASC culture medium αMEM supplemented with 5% HPL and 1% P/S (row K). Second group was exposed to ASC-CM collected from ASC culture after 48 hours (row CM). Originally, ASC-CM was also αMEM supplemented with 5% HPL and 1% P/S.

Third group was cultured in co-culture system with ASCs. Differentiated SH-SY5Y cells remained in their own well on the bottom and ASCs were added into the wells with inserts (row ASC). Both cells in third set-up, SH-SY5Y cells on the bottom and ASCs in the insert, were cultured in basic ASC culture medium αMEM supplemented with 5% HPL and 1% P/S (figure 2).

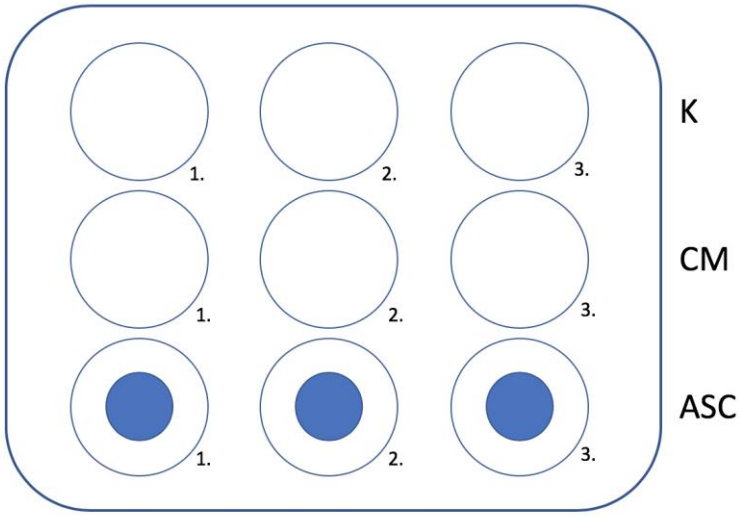

Figure 2. Differentiated SH-SY5Y cells in co-culture system with ASCs. White circles are wells containing SH-SY5Y cells. Blue circles represent inserts containing ASCs. K=5%HPL, CM=5% HPL; ASC-CM and ASC=5% HPL.

3.3.1 ASC culture, collection and production of ASC-CM

The use of ASCs is conducted in accordance with the ethic committee of the Pirkanmaa Hospital District Tampere Finland (R15161, R16036).

ASCs were cultured in density of 10 000 c/cm^2. ASC-CM is cell-free ASCs culture medium collected after 48 hours incubation in ASC culture. ASC-CM was collected and sterile-filtered.

ASCs were collected for inserts from T-75 flask ASC culture. After aspirating off old medium, cells were washed with PBS and reattached with Tryple. Cells were collected in 5% HPL medium into colonial tube, centrifuged and re-suspended the pellet into 5% HPL medium. After counting, needed amount of ASC cell-suspension was pipetted and diluted with 5% HPL medium. One insert concentration was 10 000 ASCs in the volume 500 µl of medium.

3.3.2 Co-culture with ASC

Co-culture was performed on the laminin coated 24-well plate with differentiated SH-SY5Y cells. All cells in co-cultural system were cultured in ASC basic culture medium described above. On 24-well plate one well volume is 1 ml but with insert 500 µl (insert 500 µl + bottom of the well 500 µl).

Differentiated SH-SY5Y cells were already on the bottom of the 24-well plate (see chapter 3.2.4). SH-SY5Y differentation medium DM#3 was removed from the K-row wells and ASC basic culture medium supplemented with 5% HPL was added instead. On CM-row, DM#3 was replaced with ASC-CM.

On ASC-row, after removing DM#3, 500 µl of ASC basic culture medium was added to each well. Insert was placed and 500 µl of ASC cell-suspension (10 000 cells/500 µl) was pipetted into insert.

Co-cultural system was monitored in order to study if differentiated SH-SY5Y cells survive in co-cultural conditions with ASCs. Also, part of the co-cultural plate was examined with immunocytochemistry, described in chapter 3.5, for same reasons.

3.4 Oxygen-glucose deprivation

Differentiated SH-SY5Y cells cultured on 24-well plate were exposed to OGD treatment. Preparations for OGD treatment started with replacing DM#3 culture medium for non-glucose DMEM (DMEM no-gluc. -row, figure 3) to half of the wells to mimic glucose deprivation. Another half (ND-row) was left with DM#3. Wells from these two rows, "ND" and "DMEM no-gluc." were organized into three groups: K, CM and ASC equivalent to co-cultural system. However, ASC wells did not include ASC cells yet, only differentiated SH-SY5Y cells. After preparations the plate was placed into incubator and oxygen deprivation was performed at atmosphere of 1% O_2 and it lasted four hours.

After OGD, media were changed according to co-cultural system. ASC basic culture medium supplemented with 5% HPL was changed into K-wells and ASC-wells. Into ASC-wells, ASC-CM and inserts with ASCs was added (figure 3). The density of ASCs was same as in co-cultural system, 10 000 cells/500 µl.

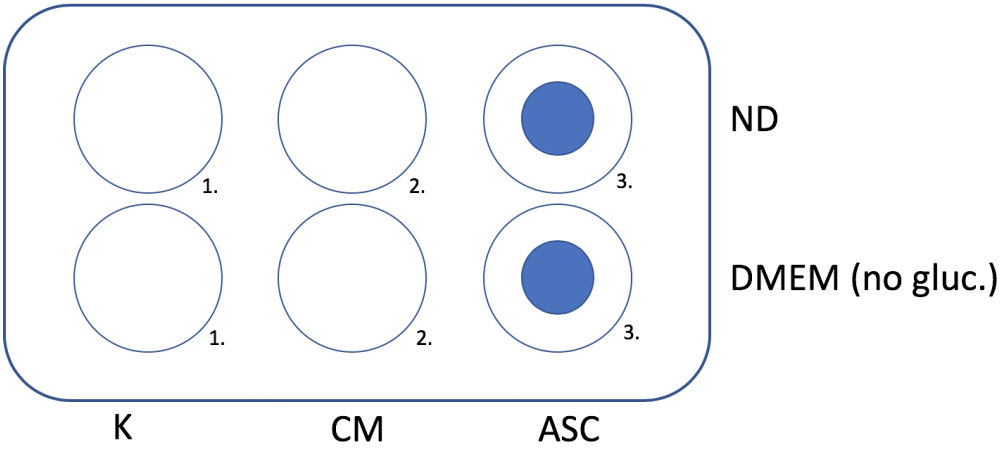

Figure 3. Differentiated, hypoxia treated SH-SY5Y culture system. Presentation is same as in figure 2 describing co-cultural system. ND and DMEM (no gluc.) pointing to culture medium character during OGD treatment.

3.5 Immunocytochemistry

3.5.1 Immunostaining for differentiated SH-SY5Y cells

Immunostaining was used in order to examine successful differentiation of SH-SY5Y cells, to examine SH-SY5Y cells in co-cultural system and also to observe possible ischemic effect of OGD treatment.

Differentiated SH-SY5Y cells were first characterized with immunocytochemistry with mature neuronal markers microtubule-associated protein 2 (MAP2) and βIII-tubulin to visualize successful differentiation. MAP2 is expressed only in neuronal cells and specifically localized in the dendrites of neuronal cells (45) whereas βIII-tubulin is specifically found in axons (46).

Differentiated SH-SY5Y cells were examined with primary antibodies anti-MAP2 (rabbit) and anti-βIII-tubulin (mouse) and anti-caspase-3 (rabbit). Used secondary antibodies were Alexafluor 488 donkey anti-rabbit (green) and Alexafluor 568 donkey anti-mouse (red). DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining (table 3).

Table 3. Used primary and secondary antibodies and nuclear stain DAPI.

Primary antibodies	Dilution	Origin	Secondary antibodies (dilution)	Emitted fluorescence	Target	Manufacturer
Cleaved caspase-3 (D175)	1:400	Rabbit	Alexafluor 488 Donkey Anti-rabbit (1:400)	green	Apoptosis marker	Cell Signaling Technology, USA
MAP2 AB 5622	1:400	Rabbit	Alexafluor 488 Donkey Anti-rabbit (1:400)	green	Dendrites	Merck KGaA, Germany (Millipore)
β-III-tubulin T8660	1:1000	Mouse	Alexafluor 568 Donkey Anti-mouse (1:400)	red	Axons	Merck KGaA, Germany (Sigma-Aldrich)
DAPI (4′,6-diamidino-2-phenylindole)	1:2000	--	--	blue	Nuclear and chromosome counterstain	

Immunostained wells were examined under Olympus IX51 fluorescence microscope.

3.5.2. Immunostaining of co-cultural and hypoxia plates

Possible ischemic effect on neuronal cells after OGD was examined with caspase 3. Caspases are family of proteins that regulate apoptosis (47). Caspase 3 can potentially be found after neurodegenerative event (48).

From co-culture plate part of differentiated SH-SY5Y cells were stained with primary antibodies anti-MAP2 and anti-βIII-tubulin to examine co-culture effect on differentiated SH-SY5Y cells. Other part from co-culture plate was stained with primary antibodies anti-βIII-tubulin and anti-caspase-3. It was assumed that caspase 3 activity on co-cultural plate is less than in hypoxia plate, if co-culture is not causing impairing effect on differentiated SH-SY5Y cells.

From OGD treated hypoxia plate three different ND-wells K, CM and ASC-CM and also, K, CM and ASC-CM wells from DMEM (non gluc.) -culture conditions were stained with primary antibodies anti-βIII-tubulin and anti-caspase-3 (table 4). Caspase 3 activity was assumed to be visible if neuronal

cells suffered from ischemic injury and presupposed to be visible especially in DMEM (non gluc.) -wells.

Table 4. Primary antibodies in co-culture plate (1 and 2) and hypoxia plate (3).

1. Co-culture plate	2. Co-culture plate	3. Hypoxia plate
K βIII-tubulin+MAP2	**K** βIII-tubulin+Caspase 3	**ND** βIII-tubulin+Caspase 3
CM βIII-tubulin+MAP2	**CM** βIII-tubulin+Caspase 3	**DMEM (non gluc.)** βIII-tubulin+Caspase 3
ASC βIII-tubulin+MAP2	**ASC** βIII-tubulin+Caspase 3	

3.6 CyQuant analysis and statistics

CyQUANT is highly sensitive, fluorescence-based assay where CyQUANT GR-dye binds to cellular nucleic acids. Fluorescence emission correlates linearly with cell number on the plate. (49,50)

In CyQUANT assay cells were frozen for efficient lysis result. Thawed cell pellet was resuspended with CyQUANT GR-dye and cell-lysis buffer and cell containing sample dilution was added to microplate in volume 200µl per well. Measurement of fluorescence was conducted under microplate fluorescence reader (Victor^2, 1420 Multiplate Counter by Wallac).

CyQUANT assay is usable in proliferation studies and in the present study it was used to examine ASCs´ effect on SH-SY5Y derived neuronal cell proliferation. Each three well from each row (classes K, ASC, ASC-CM) from co-culture plate (figure 2) were measured with three parallel values, totally 9 values per one class. Distilled water was used as blank and it was also measured with three parallels.

As a statistic analysis, Shapiro-Wilk test was used to test the normality and Kruskal-Wallis H-test was used to determine whether there was statistically significant difference between groups. Groups were then compared with independent sample variable tests for non-parametrical testing to describe the difference. Statistics were conducted with IBM SPSS Statistics (version 25.0). Result is considered statistically significant when $p<0.05$ with CI 95%.

4 RESULTS

4.1 SH-SY5Y differentiation

SH-SY5Y differentiation was successful. SH-SY5Y cells showed changed morphology during differentiation from blunt, clustered cells to neuronal-like cells. Typical neuronal projections and connections between cells were visible under microscope as shown in figure 4.

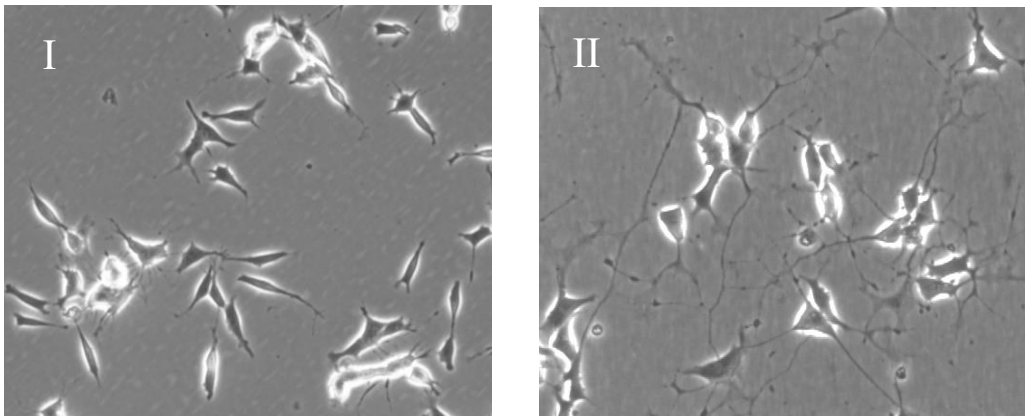

Figure 4. SH-SY5Y cells at the beginning of differentiation on day 1 (I) and at the end of differentiation on day 22 (II). Magnification x10.

Figure 5 shows immunofluorescence staining of mature neuronal markers MAP2 in dendrites and βIII-tubulin in axons in differentiated SH-SY5Y cells. Dendrites are shown in green and axons in red. DAPI is nuclear stain that stains nuclei blue. Markers indicate that SH-SY5Y cells differentiated into mature neurons.

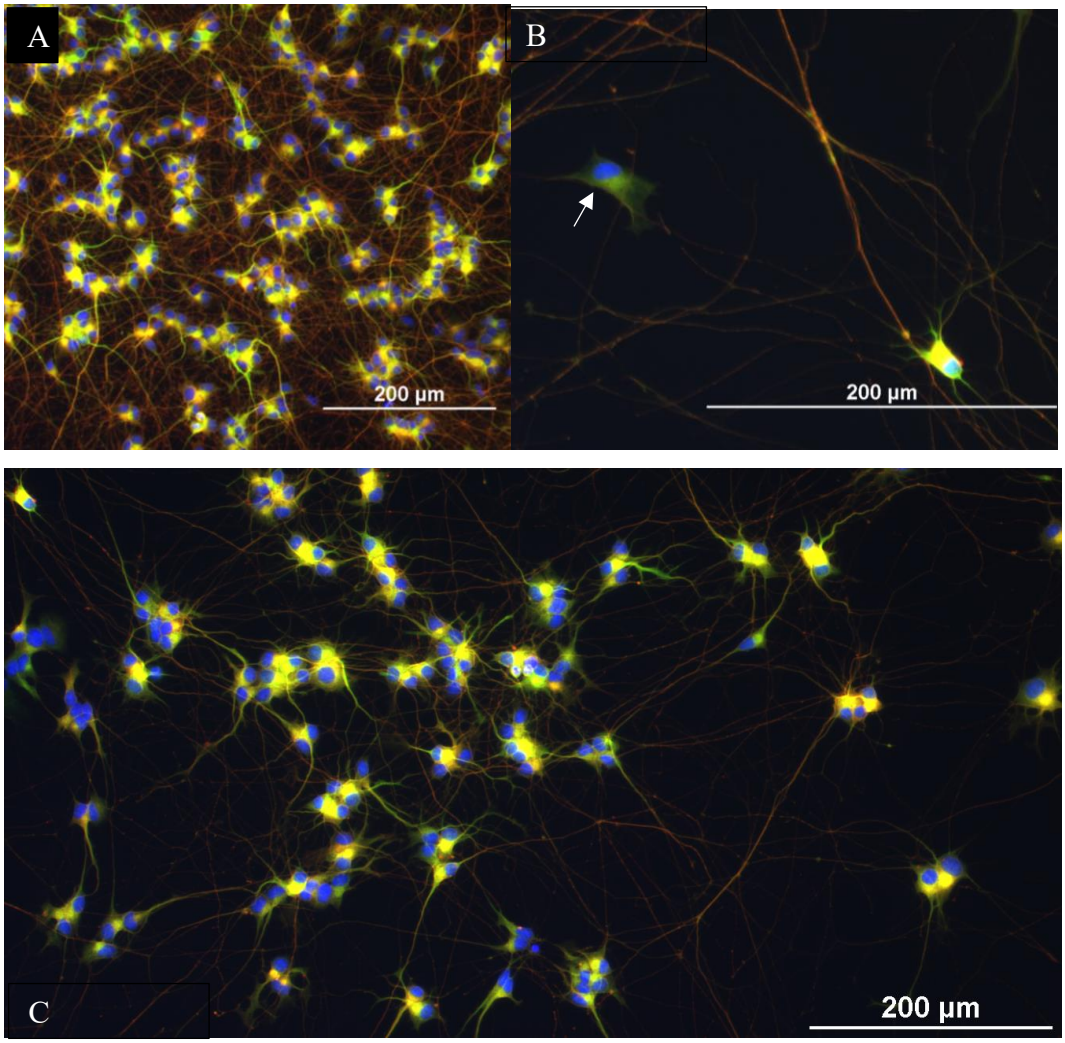

Figure 5. Fluorescence stained differentiated SH-SY5Y. Colors: red = β-tubulin-III, green = MAP2, blue = DAPI, nucleus. In figure (B) there is epithelial like SH-SY5Y cell on the background pointed by the arrow.

4.2 Co-cultural system and CyQUANT analysis

Microscopic examination of differentiated SH-SY5Y cells cultured in ASCs´ basic growth medium, in ASC-CM or in co-cultural system with ASCs showed no change on cell viability or decrease of proliferation. Instead, differentiated SH-SY5Y cells seemed to proliferate when cultured in ASCs` basic growth medium. In addition, differentiated SH-SY5Y cells survived well in co-cultural system despite the insert.

Immunocytochemistry assay showed mature neurons as MAP2 and βIII-tubulin was detected from K, CM and ASC wells. Differentiated SH-SY5Y cells seemed undisturbed in co-cultural system with ASCs, ASC basic growth medium or with ASC-CM.

CyQUANT analysis results were measured as an amount of fluorescence. Fluorescence correlates linearly with the amount of DNA on the plate. Figure 6 shows boxplot of data in logarithmical scale from CyQUANT measurements with blank deducted. One boxplot figure presents three wells with three parallel measurements with total number of samples being nine per culture condition.

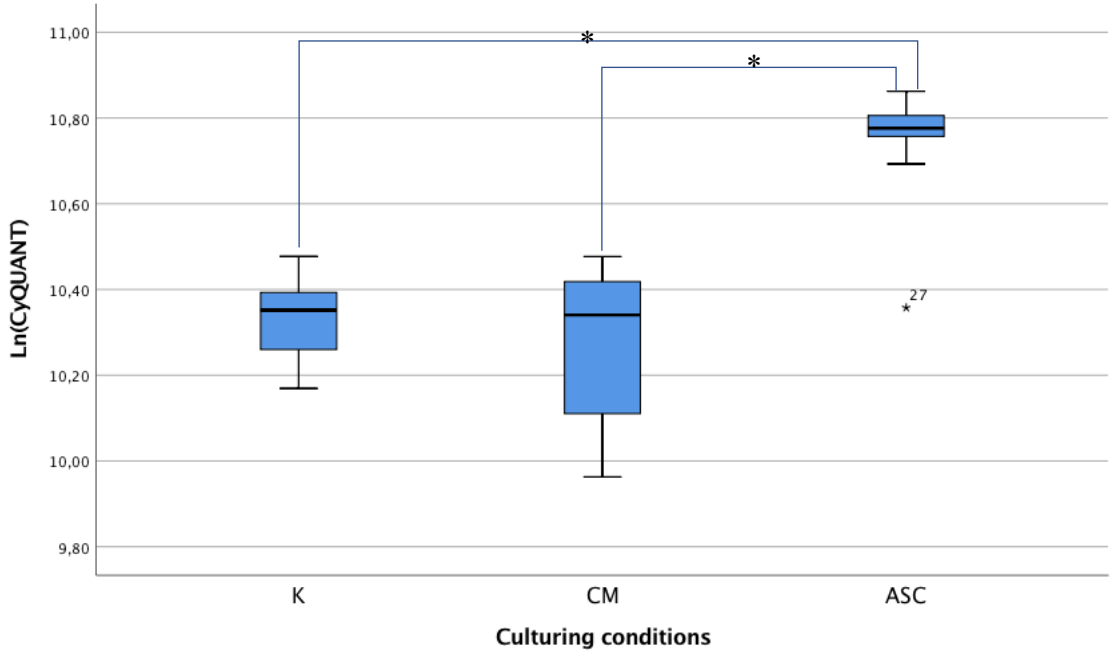

Figure 6. CyQuant analysis results from co-cultural plate presented in logarithmical scale. *p<0.05 (95% CI) picturing statistically significant difference between groups tested with independent sample variable tests for non-parametrical testing.

Test of normality conducted with Shapiro-Wilk test resulted that K and CM were normally distributed, but ASC did not reach normal distribution (p(K)=0.867, p(CM)=0.073, p(ASC)=0.001). Therefore, statistically significant difference between groups was tested with nonparametric Kruskal-Wallis H-test and comparison between groups was tested with independent sample variable tests for non-parametrical testing.

Kruskal-Wallis H-test resulted that there was statistically significant difference between groups (H=14.169, p<0.001). Independent sample pairwise comparison resulted that the difference occurs when comparing K and CM to ASC (p<0.01, table 5), indicating the statistically significant difference is in comparison with ASC.

Table 5. Results from independent sample pairwise comparison *p*(I).

Culture condition	Comparison	*p*(I)
K	CM	1.00
	ASC	<0.01
CM	K	1.00
	ASC	<0.01

CyQUANT analysis and statistical test results describe that SH-SY5Y cultured with ASCs in the insert in ASC well differs from other culture conditions, K and CM. The amount of fluorescence measured from ASC well was notably higher comparing to K and CM and statistics support this conclusion. Results indicate that differentiated SH-SY5Y cells survived well in all different culture conditions, though survival could be considered being best in ASC well. Conclusion is that differentiated SH-SY5Y cells can be examined and tested further with ASCs, ASC-CM or with ASC basic growth media.

4.3 Oxygen-glucose deprivation

Differentiated SH-SY5Y cells were exposed to OGD treatment for four hours. After treatment, apoptosis and possible cell injuries were examined with immunocytochemistry using βIII-tubulin and caspase 3 antibodies.

βIII-tubulin was visible on both plates but caspase 3 could not be confirmed to have actual activity. Co-cultural plate that had not been exposed for OGD treatment was used as negative control for absence of caspase 3 activity. It was assumed that caspase-3 is less visible on plate that is not exposed to OGD treatment. However, the plate showed green emission, but it might have been artifact as it was bright and partly outside cells. Green light emission was detected from OGD treated plate also, but it might have been the same artifact as in negative control plate (figure 7).

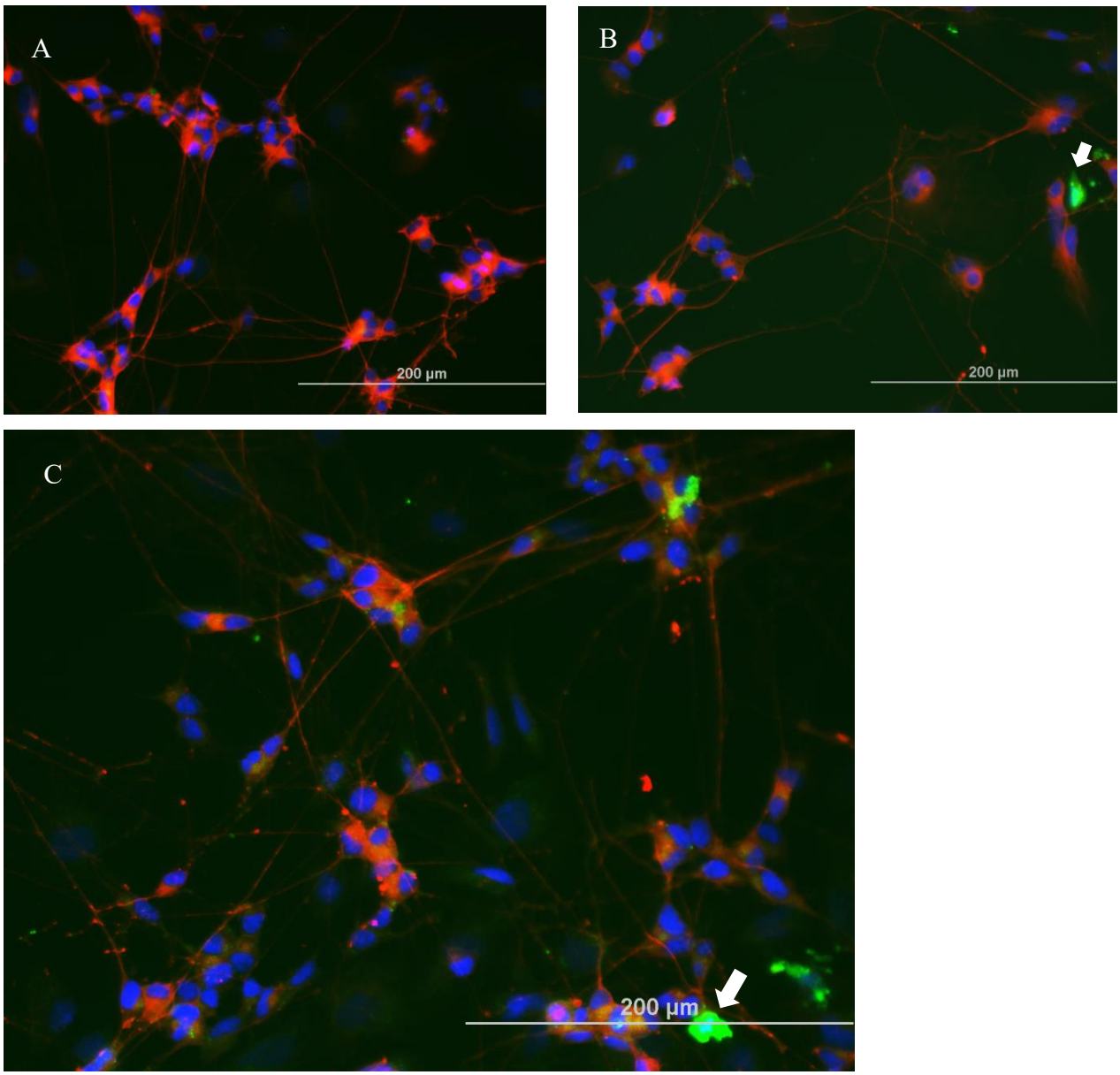

Figure 7. Fluorescence stained differentiated SH-SY5Y cells from OGD treated plate containing no glucose (A and B) and negative control (C) from co-cultural plate. Magnification x20. Colors: nucleus = blue, β-tubulin-III = red, caspase 3 = green. Arrows pointing possible artefacts in figures B and C.

5 DISCUSSION

Properties of stems cells from different sources have been studied in order to find new therapeutic methods to improve neuronal recovery after stroke. Studies suggest that ASCs have an effect on neuronal cell regeneration and they potentially secrete neuroprotective factors. (20,21,51) ASCs may possess a new treatment option of regenerative medicine (52) and for example, for ischemic stroke in the future (8,11). Yet there are limitations in clinical trials of current adult stem cell therapies such as finding an optimal time window for stem cell transplantation and possible stem cell -mediated side effects such as tumor formation (11).

Stem cell therapies are experimental treatment option. Therapeutic efficacy of stem cells is not yet fully proved in in vitro and in vivo studies. Furthermore, for example a proper mode of delivery needs to be tested for cell therapies. Stem cells derived from different sources have been examined as an option for regenerative medicine and especially ASCs have shown a great potential comparing to stem cells from other sources. ASCs valuable characteristics are their easy accessibility, they have been proofed to be safe in animal models and their proliferation capacity is high comparing to for example bone marrow derived stem cells. Although, the mode of delivery and therefore the migration to the damaged site is one of the main issues concerning ASC treatments and possible tumorgenicity requires more studies. (42)

Delivery of stem cells have been studied in animal models in order to test stem cell therapies in vivo and also to screen an effective mode of delivery. Intravenous autologous ASC infusion for rats affected by in vivo stroke resulted in decreased infarct lesion size and to improved neurological function (16). Other study showed similar results. Infarct lesion size in rats decreased notably and in correlation with the dose of stem cells delivered intravenously. Largest dose of stem cells resulted to most decreased lesion size.(53) Though this study used immortalized human mesenchymal stem cells from healthy adult bone marrow, and not ASCs. In other study, in vivo ischemic model conducted by occlusion of middle cerebral artery in rats showed that intracerebral injection of human ASCs into lateral ventricle was efficient improving the symptoms of stroke compared to ischemic group that did not receive ASCs (54).

However, intravenous delivery is preferred as option for humans as it is less invasive than intracerebral/intracranial injection (10,42,55). In one clinical trial MSC derived from umbilical cord were delivered intra-arterially with catheter near the lesion. Intra-artery infusion proofed to be safe

method, but further conclusions from treatment results are difficult to comprise because study had only three ischemic stroke patients and lacked placebo control. (56) Also, intravenous infusion was verified as a safe route for administration of multipotent adult progenitor cells for stroke patients (41).

Human origin cells are more preferable to be used in studies in order to study stroke mechanism and to find new therapeutic methods for stroke in humans. SH-SY5Y cells possess properties of human neuronal cells compared to animal origin cells when differentiated. Therefore, these cells might be more adequate for testing human neuronal cell mechanisms and ischemic response than often used rodent models. (25,57) SH-SY5Y cells express neuronal differentiation markers and reflect mature neuronal phenotype after RA/BDNF treatment and also developed more elongated neurites compared to undifferentiated SH-SY5Y cells. (28) These results suggest that SH-SY5Y cells are considerable option for neuronal cell ischemia studies in vitro.

Modelling ischemic stroke in vitro is a key in the investigations aiming to find new effective therapy for treating stroke. In vivo studies often use rodent models, but their neuronal functions differ from human. (39) Although, in vivo studies have offered promising results concerning the efficacy of stem cell treatment for neurodegenerative events. Both the outcome afterwards and also examination of the infarct site has shown that stem cells and therefore ASCs might be potent treating option for stroke. (10,11,17,58)

In cell cultures OGD treatment is a potent way to induce ischemic like condition for neuronal cells in vitro as it mimics events of stroke. OGD can be carried out with enzymatic or chemical method. Conditions mimicking ischemia can result to neuronal cell damage with cell swelling and apoptosis or excitotoxic neuronal cell death. In the future, OGD treatment and in vitro ischemia studies could aim to even more brain-like conditions with synthetic 3D platforms for the cells and with systems including microfluid techniques to model neuronal architecture. (39) Improvements in in vitro studies can help to understand clinical trials results better and to improve clinical trials towards more efficient treatment results.

OGD treatment times vary between studies. In one study OGD treatment was 16 hours (37) and in other 5 hours (38). One study suggests that OGD treatment time with only 90 minutes induces apoptosis in cell culture although the maximal effect was reached 12 hours after the treatment (59). In the present study OGD treatment time was only 4 hours. The range for treatment time in other studies is quite broad and adjustments need to be studied in order to find proper method for in vitro

ischemic model. It should be noted that SH-SY5Y cells are not only neuronal cell population used in studies and results might differ because of the origin of neuronal cells.

Visualization and localization of mature neuronal markers can be carried out with immunostaining showing MAP-2 and β-tubulin-III. MAP-2 is widely used label in studies examining neuronal cells with immunocytochemistry (60-64). β-tubulin-III is also suitable for studying mature neuronal cell markers but it might be less used compared to MAP2 (65,66).

Caspase-3 is considered as executer of apoptotic cell death in central nervous system. In vitro ischemia caused by OGD treatment can lead to increased caspase-3 activity. Though caspase-3 activity was proofed to associate with rat cultured septo-hippocampal neuronal cell death, the immunoreactivity in immunocytochemical assay showed only robust results. (67) In one study caspase 3 activity was significantly lower in non-ischemic control group compared to in vivo ischemia group on rat model (16), but test was conducted measuring caspase 3 mRNA rather than visualizing it with immunocytochemistry. However, densitometric quantification of caspase-3 in motor-neuron-like cells showed increase in injured neuronal cells and moreover immunocytochemistry supported the result. (68) Also, in another study caspase-3 staining was visible showing neurodegeneration in mice-origin hippocampal neurons after glutamate-treatment. In control culture that was not treated with glutamate, caspase-3 activity was 8% indicating that neuronal cell cultures show little degeneration all the time and even in the absence of external stimuli. Activity of caspase-3 was not visible on astrocytes or microglia so it was limited to damaged neuronal cells. (63)

Antibodies MAP-2 and βIII-tubulin chosen for this study were efficient observing mature neuronal cells, but caspace-3 staining did not bring assumed apoptosis visible. OGD treatment time may have been too low as caspase-3 activity was not detected. Since there was no positive control for apoptosis, it remains unclear whether there was an absence of caspase-3 due to short hypoxia time or poor staining result is a second to non-functioning antibody.

All in all, adult stem cell research in the field of regenerative medicine has shown promising results but straight conclusions from in vitro studies cannot be done concerning effects in vivo (9,13). Only few clinical trials have been conducted and conclusions can be made more from delivery methods and their safety rather than improvements of infarct site due to low quantity of patients and lack of placebo control. (55,56) Also, further studies can be used for development of potency assays in order to evaluate parameters for cell treatment product`s biological effect and quality. (44) More

investigations concerning stem cell therapies for stroke needs to be done before moving to clinical trials. Cell culture studies are relevant prior in vivo studies and clinical investigations.

6 CONCLUSIONS

The present study provides the following conclusions according to objectives:

1. Differentiation protocol of SH-SY5Y cells into mature neuronal cells proofed to be successful. SH-SY5Y cell morphology was changed during differentiation protocol towards neuronal cell morphology and cells expressed mature neuronal markers in immunocytochemical assay.

2. Differentiated SH-SY5Y cells survived well in co-cultural system in all three different environments. Immunocytochemical assay showed mature neuronal markers and CyQUANT analysis results disclosed that cells proliferated in co-cultural systems. Results indicate that SH-SY5Y derived neuronal cell culture can be examined together with ASCs and is therefore potent for studying for example neuronal in vitro ischemia and ASCs` effect on neuronal cell injuries. Different culturing media and the presence of ASCs did not interfere SH-SY5Y derived neuronal cells.

3. OGD treatment performed for 4 hours was not sufficient to cause damage on SH-SY5Y derived neuronal cells. Immunocytochemical assay with mature neuronal markers MAP-2 and βIII-tubulin showed intact neuronal cells with no visible damage under fluorescence microscope. Also, the apoptosis marker caspase-3 was not visible in immunocytochemical assay.

   In conclusion causing in vitro ischemia with 4-hour OGD treatment was not successful and treatment time should be optimized in the future studies. Also, OGD treatment could be repeated with different time frames and parallel cultures to achieve ischemic results in order to examine ASC paracrine effect on damaged neuronal cells. Aim of this study was not fully encountered, as neuronal cell damage was not achieved with 4-hour treatment.

Future cell culture studies utilizing co-culture systems could investigate ASCs´ paracrine effect on SH-SY5Y derived neuronal cells under in vitro ischemic model. Also, it can be examined whether ASCs are actually needed in the infarct site or is the conditioned medium and its substances suitable option for therapy product. In the future treating stroke with stem cell-based medicine can be possible. Still more work needs to be done in order to develop a human in vitro stroke model and to find proper dose and mode of medication, whether it is injected stem cells, or they secreted products.

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